Age and DRD4 Genotype Moderate Associations Between Stimulant Treatment History and Cortex Structure in Attention-Deficit/Hyperactivity Disorder

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Title: Age and *DRD4* genotype moderate associations between stimulant treatment history and cortex structure in ADHD

Running title: *DRD4*- and age-specific stimulant effects

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Objective: Attention-deficit/hyperactivity disorder (ADHD) has been associated with dopaminergic imbalance and subtle volume reductions in the brain. Stimulants acutely enhance dopaminergic neurotransmission. Long-term effects of chronic manipulation of the dopaminergic system on brain structure remain poorly understood; they could be beneficial or unfavorable, and may be moderated by common genetic variants and/or age.

Method: In a large observational cohort study (N_{ADHD}=316), we evaluated the effects of cumulative stimulant treatment, genotype (for *DAT1* haplotype and *DRD4* variants), and treatment-by-genotype interactions on striatal, frontal, and hippocampal volumes, as well as their interactions with age.

Results: We found no main effects of treatment. Associations between treatment and bilateral frontal and left hippocampal volume depended on *DRD4* genotype and age. At younger age and lower treatment-levels, but not at younger age and higher treatment levels, carriers of the *DRD4* 7R-allele showed decreased frontal cortex volumes. At older age, both carriers and non-carriers showed lower frontal volumes irrespective of treatment history. Left hippocampal volume was similar to controls at average treatment levels, and increased with treatment only in carriers of the *DRD4* risk allele and at younger age. No interaction effects were found in the striatum.

Conclusions: Carriers of the *DRD4* risk allele may at younger age be sensitive to cortical remodeling after stimulant treatment. The cross-sectional nature of our study warrants cautious interpretation of age effects. Our findings, although of small effect size, may ultimately contribute to optimal care for individuals with ADHD.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) has been associated with widespread subtle changes in brain structure. Total gray matter volume is reduced by 2-3% in children with ADHD compared to typically developing children, with more pronounced reduction and atypical age-related changes in the frontal-striatal system.[1-4] The striatum and its frontal connections are rich in dopaminergic neurons, and ADHD symptoms are thought to, at least partially, stem from dopaminergic and noradrenergic imbalances.[5] Stimulants such as methylphenidate enhance dopaminergic and noradrenergic neurotransmission by binding to the dopamine transporter, thereby inhibiting presynaptic dopamine reuptake, and increasing extracellular dopamine availability.[6]

Long-term effects of stimulant treatment on the developing brain remain poorly understood. Although several studies have suggested fewer structural abnormalities in individuals with ADHD after long-term stimulant treatment,[e.g.2,7] findings are equivocal. Meta-analyses found larger (thus more normative) striatal volumes in studies including a higher percentage of stimulant-treated patients compared to studies with lower percentages.[1,4] However, recent large-scale original studies did not find evidence of structural normalization in the striatum.[3,8] In the frontal cortex, disproportionate cortical thinning has been found in non-treated children but not in stimulant-treated children with ADHD,[7] while others have found reduced middle frontal cortex volumes in stimulant-treated compared to stimulant-naive patients.[9] Prior analyses of the current sample found no treatment effects on frontal cortical thickness.[10] Thus, conclusive evidence of long-term treatment effects on frontal-striatal brain structures is missing.

Genetic make-up may predispose potential brain changes after stimulant treatment. The 3'-untranslated region (3'UTR) of the gene encoding the *dopamine transporter* (*SLC6A3/DAT1*)

contains a variable number of tandem repeat (VNTR) polymorphism influencing presynaptic dopamine transporter density, especially in the striatum where gene expression is high. The 9- and 10-repeat alleles are most frequently encountered in the population. In children and adolescents, the 10-repeat allele has been associated with increased risk of ADHD,[11] smaller striatal volumes,[12] and distinct striatal activity patterns,[13,14] but not with clinical treatment response.[15,16] Recent studies have performed association analyses based on a haplotype of the 3'UTR VNTR and a second VNTR of the *DAT1* gene located in intron 8.[17] The 10-6 haplotype (10-repeat allele in the 3'UTR VNTR, 6-repeat allele in the intron 8 VNTR) has been identified as the risk haplotype for ADHD in children and adolescents,[18] whereas the 9-6 haplotype has been associated with adult ADHD.[19] Associations between the *DAT1* haplotype, stimulant treatment, and brain structure have not yet been investigated.

A second dopaminergic gene, the *dopamine receptor D4 (DRD4)* gene, encodes the postsynaptic dopamine D4 receptor, and is highly expressed in the frontal cortex and hippocampus.[20,21] The 7-repeat allele of a VNTR in exon 3 (*DRD4* 7R) has been identified as the risk allele for ADHD, but has also been associated with better clinical outcome in late adolescence.[22] In ADHD-enriched samples, carriers of the 7-repeat allele have shown reduced frontal cortex volume and thickness.[22,23] Moreover, *DRD4* genotype modulated prefrontal cortex activation during various tasks.[24,25] In the current sample, *DRD4* genotype and social environment together, but not *DRD4* genotype alone, influenced prefrontal cortex activation during response inhibition (unpublished results). It has been suggested that the *DRD4* polymorphism may be linked to attention problems.[26] Most treatment studies failed to predict clinical treatment response from *DRD4* 7R-carriership,[e.g.27,28] although modest genotype-by-dose interaction effects have been reported.[e.g.29,30]

With *DAT1* and *DRD4* genes affecting presynaptic dopamine transporters in the striatum and postsynaptic dopamine receptors in the frontal cortex and hippocampus, respectively, inter-individual genetic differences may predispose treatment effects in these brain regions. Pharmacological neuroimaging studies have shown different acute striatal responses to methylphenidate in individuals with different *DAT1* 3'UTR genotypes.[31,32] Furthermore, the dopaminergic system undergoes changes during development, hence long-term treatment and genetic effects on brain structure may be different at different ages. Rapidly developing brain regions are particularly sensitive to external influences such as stimulant exposure.[33] Support for age-dependent long-term stimulant treatment effects comes from animal studies showing striatal volume reduction and hippocampal shape deformations after chronic juvenile exposure but not following treatment in adulthood.[34,35] Long-term structural changes after stimulant treatment may reflect dopamine-dependent long-term plasticity, a process of structural remodeling to which the hippocampus is known to be particularly sensitive.[36] Thus, differential neural susceptibility to acute methylphenidate effects may translate into differential sensitivity to long-term stimulant treatment effects on brain structure.

Identifying sources of neural sensitivity to long-term treatment effects is important, and may ultimately influence therapeutic decisions. Here, we investigated associations between stimulant treatment, genetic predispositions, age, and brain structure. We hypothesized that stimulant treatment would be associated with larger (more normative) striatal volume, and that this association would be more pronounced in *DAT1* 10-6 risk haplotype carriers and at younger age. Second, we hypothesized that stimulant treatment would be associated with larger frontal and hippocampal volume, especially in *DRD4* 7R-allele carriers and at younger age. We investigated these hypotheses in a large cross-sectional sample of children, adolescents and young adults with ADHD. The current paper adds to prior studies of our group that focused on

case-control differences in brain structure.[3,10] Inter-individual differences in neural sensitivity to long-term treatment effects, due to age and/or genetic make-up, have not been addressed in prior studies in our sample.

Method

Participants

Participants with ADHD (n=316, mean age=17.2 years, 69.3% male) and control participants (n=187, mean age=16.5 years, 52.4% male) were selected from the Dutch family-based follow-up phase (NeuroIMAGE) of the International Multisite ADHD Genetics (IMAGE) study. The protocol included diagnostic interviews, questionnaires for participants, parents, and teachers, DNA collection, and a magnetic resonance imaging (MRI) session, taking place at two testing sites in The Netherlands (Amsterdam and Nijmegen). Informed consent was signed by participants ≥ 12 years and parents of participants < 18 years. The study was approved by the ethical committee of each site. ADHD diagnosis and type (inattentive, hyperactive/impulsive, or combined type), ADHD severity, and axis-I comorbidity were obtained from a diagnostic interview[37] and Conners ADHD questionnaires,[38-40] rated while participants were off-medication. Controls were required to have no first-degree relative with psychiatric problems, i.e., unaffected siblings of participants with ADHD were excluded. All participants were of European Caucasian descent. For a detailed description of inclusion and diagnostic criteria, see[41].

Treatment history

Pharmacy transcripts and self/parent-report questionnaires were combined to assess treatment history. For each stimulant-treated participant (immediate and/or extended release

methylphenidate preparations and/or d-amphetamine preparations), a dose-by-age trajectory from age=0 to age at scan was reconstructed (Figure 1). The area under the curve equals cumulative stimulant intake. Cumulative intake was divided by participant's age minus 2.3 (minimum stimulant start age within the cohort) to obtain an age-adjusted treatment variable (CSI_{ADJ}) in mg/year which was subsequently standardized into a z-score. One extreme outlier ($Z_{CSI\text{-}ADJ}>4$) was excluded (details available online, S1). Since CSI_{ADJ} is a composite parameter capturing dose, start age, treatment duration, and time since last treatment, these alternative parameters were evaluated post-hoc.

Genotyping

DNA was extracted from blood or saliva samples (for details, see[42]). No deviations from Hardy-Weinberg Equilibrium were found (p_{DRD4}=0.15, $p_{DAT1\text{-}3'UTR}$=0.78, $p_{DAT1\text{-}INTRON\ 8}$=0.55). *DAT1* haplotypes were calculated using the HaPloStats package (R version 2.12.0).[43] Participants with zero, one, or two copies of the *DAT1* 10-6 risk haplotype were distinguished. We performed pairwise testing to avoid imposing a linear model (0*vs*1 copy, 0*vs*2 copies, and 1*vs*2 copies). For the *DRD4* 7R-allele, we differentiated between non-carriers (0 copies) and carriers (1 or 2 copies), in line with the literature. Allele frequencies are in Table 1.

Magnetic Resonance Imaging

MRI data was acquired on two 1.5T Siemens scanners (Siemens, Germany), equipped with product 8-channel phased-array head coils using equivalent acquisition parameters. The session consisted of multiple acquisitions, including two T1-weighted 3D-MPRAGE scans (TI=1000 ms, TR=2730 ms, TE=2.95 ms, FA=7°; 176 sagittal slices, 1x1x1 mm voxels). For each participant, the structural acquisition of highest quality was selected by visual inspection,

accepting only scans with no/mild distortions.[44] For data quality and compatibility between sites, see[41].

Striatal and hippocampal volumes were obtained with FMRIB's Integrated Registration and Segmentation Tool (FSL FIRST).[45] As our hypothesis regarding treatment effects was the same across striatal structures, striatal volume was calculated as the sum of caudate nucleus, putamen, and nucleus accumbens volume; individual structures were evaluated only post-hoc. Frontal cortex volume was derived by multiplying cortical thickness and surface area of the medial and lateral orbitofrontal, inferior frontal, caudal and rostral medial frontal, superior frontal, and frontal pole cortex, reconstructed using the automated Freesurfer pipeline.[46,47] Total brain volume (TBV) was acquired using SPM (VBM8.1 toolbox, http://dbm.neuro.uni-jena.de/vbm/) as the sum of gray and white matter tissue probability maps.

Analyses

All analyses were performed separately for three regions of interest (ROI; striatum, frontal cortex, hippocampus) in two hemispheres (left, right). ROI volumes were predicted from CSI_{ADJ}, genotype (*DAT1* haplotype for striatum, *DRD4* genotype for frontal cortex and hippocampus), and CSI_{ADJ}-by-genotype interaction, in linear mixed effects models with covariates gender, site, age, age^2, TBV, and a random intercept per family to correct for relatedness within the sample ('initial models'; volume ~ $\alpha + \beta$*covariates + β*CSI_{ADJ} + β*genotype + β*CSI_{ADJ}*genotype). Age and CSI_{ADJ} were standardized, such that main effects of the predictors of interest are conditional to average age and treatment; non-genotype effects are also conditional to a reference category, i.e., *DAT1* 10-6 homozygotes and *DRD4* 7R-carriers.

Next, the initial models were extended to allow interactions with age ('age-interaction models'; volume ~ $\alpha + \beta$*covariates + β*CSI_{ADJ} + β*genotype + β*CSI_{ADJ}*genotype +

β*age*CSI_{ADJ} + β*age*genotype + β*age*CSI_{ADJ}*genotype + β*age^2 *CSI_{ADJ} + β*age^2*genotype + β*age^2*CSI_{ADJ}*genotype). Non-significant interaction terms were dropped from the model one-by-one, each time eliminating the highest-level and least-predictive interaction term and re-estimating the regression coefficients. Predictors and covariates from the initial model and lower-level interaction terms conditional to significant higher-level interaction terms were never removed. Alpha was divided by six (α=0.05/3/2=0.008).

Clinical variables associated with stimulant treatment (e.g., severity) may drive spurious associations between brain volume and CSI_{ADJ}. Therefore, each variable associated with CSI_{ADJ} was post-hoc evaluated as a potential confounder. First, the confounder (and its interactions with age and genotype) was tested in a model identical to the significant CSI_{ADJ} model. If significant, CSI_{ADJ} and the confounder (and their age- and genotype-interactions) were modeled competitively. The same procedure was adopted to disentangle treatment parameters contributing to CSI_{ADJ}, i.e., non-adjusted cumulative intake, active versus past treatment, start age, treatment duration, and time since last treatment.

Case-control comparisons of subcortical volumes[3] and frontal cortex structure[10] have previously been reported. In the current study, controls served only to estimate reference volumes. All other analyses are based on participants with ADHD only. Since the full control sample differed from the ADHD sample in terms of age (M_{HC}=16.5, M_{ADHD}=17.2), gender ($Male_{HC}$=52.4%, $Male_{ADHD}$=69.3%), and site ($Nijmegen_{HC}$=38.5%, $Nijmegen_{ADHD}$=56.3%), reference volumes were also estimated for a group-matched subsample of controls (n=151).

Results

The majority of participants with ADHD were male (n=219, 69%), and most had inattentive (n=141, 45%) or combined type ADHD (n=138, 44%). Age ranged from 8 to 28 years (M[SD]=17.2[3.4] years). Comorbidities included oppositional defiant disorder or conduct disorder (n=97, 31%), anxiety/depression (n=11, 4%), and tic disorders (n=3, 1%). Age-adjusted cumulative stimulant intake ranged from 0 mg/year (treatment-naïve, n=38, 12%) to 15766 mg/year. Treatment start age ranged from 2.3 to 20.6 years, and 146 participants (46.2%) were on active stimulant treatment within three months prior to study participation. Figure 1 shows treatment trajectories over time per age quartile. At older age, more participants had ceased treatment. Eighty-one participants (25.6%) had been treated with non-stimulant psychoactive medication.

CSI_{ADJ} was higher in participants with combined type compared to inattentive or hyperactive/impulsive type ADHD (p=0.001). Furthermore, CSI_{ADJ} was marginally associated with *DAT1* 10-6 haplotype ($M_{1COPY}>M_{0COPIES}>M_{2COPIES}$, p=0.029). CSI_{ADJ} was not associated with parent-rated inattention or hyperactivity/impulsivity symptoms, IQ, SES, *DRD4* genotype, comorbidity, or non-stimulant medication. As expected, CSI_{ADJ} correlated positively with treatment duration, and negatively with start age and time since last treatment, and was higher in participants on active treatment compared to those who had discontinued (details available online, S1).

Striatal volume and DAT1

Left striatal volume was reduced in participants with ADHD carrying one 10-6 risk allele compared to 10-6 homozygotes ($M_{0COPIES}$=10.30 mL, M_{1COPY}=9.89 mL, $M_{2COPIES}$=10.14 mL; p_{1vs2}=0.005, p_{0vs1}=0.018, p_{0vs2}=0.352; effect size β[95% confidence interval]$_{1vs2}$=0.243[0.075-0.410]), with a similar trend on the right (p_{1vs2}=0.030, p_{0vs1}=0.145, p_{0vs2}=0.719). This effect was

most pronounced in the left putamen (p=0.009) and accumbens (p=0.013), and less prominent in the caudate (p=0.090). Covariates site, gender, and age or age^2 were not associated with striatal volume, but, as expected, total brain volume was (Table S2, available online). Participants with and without ADHD did not differ with regard to striatal volume (p_{LEFT}=0.531; p_{RIGHT}=0.531). Treatment (CSI_{ADJ}) was not associated with left or right striatal volume as a main effect, nor in interaction with *DAT1* haplotype, age, or age^2 (Table 2).

Frontal cortex volume and DRD4

In the initial models, neither CSI_{ADJ}, *DRD4* genotype, not their interaction was associated with frontal cortex volume (Table 2; Table S2, available online). When age and age^2 were allowed to interact with stimulant treatment and genotype in the age-interaction models, however, significant age^2-by-CSI_{ADJ}-by-*DRD4* interaction effects were found in both hemispheres (p_{LEFT}=0.003, β[CI]=0.187[0.062-0.311]; p_{RIGHT}<0.001, β[CI]=0.220[0.093-0.346]; Figure 2). At younger age (plotted at 1SD below the mean, 13.9 years), frontal cortex volume increased with increasing CSI_{ADJ} in carriers of the 7R-allele. No such association was found in carriers of the 7R-allele at older age (plotted at 1SD above the mean, 20.5 years), nor in non-carriers at older or younger age. As a consequence of the three-way interactions, the lower-level age^2-by-CSI_{ADJ} interaction effect reached significance as well, as did the age^2-by-*DRD4* interaction effect on right frontal cortex volume (Table 2).

CSI_{ADJ} was higher in combined type ADHD compared to inattentive or hyperactive/impulsive type. Models were re-estimated replacing CSI_{ADJ} with ADHD-type. Age^2-by-ADHD-type-by-*DRD4* reached nominal significance in both hemispheres for inattentive versus combined type ADHD (p_{LEFT}=0.030, p_{RIGHT}=0.045); when CSI_{ADJ} and ADHD-type (and their interactions with age and genotype) were modeled competitively, the age^2-by-CSI_{ADJ}-by-

DRD4 interaction term remained significant (p_{LEFT}=0.002, p_{RIGHT}=<0.001), while the age^2-by-ADHD-type-by-*DRD4* interaction term was marginally significant (inattentive versus combined type, p_{LEFT}=0.011, p_{RIGHT}=0.009; hyperactive/impulsive versus combined type, p_{LEFT}=0.062, p_{RIGHT}=0.044).

Finally, frontal cortex volume was reduced in participants with ADHD compared to control participants at trend level (right: M_{ADHD}=81.53 mL, $M_{CONTROL}$=82.78 mL; p=0.010; left: M_{ADHD}=82.24 mL, $M_{CONTROL}$=83.11 mL; p=0.073). Volume reduction in the right frontal cortex was significant when compared to the matched control group (Table S3, available online).

Hippocampus volume and DRD4

Neither CSI_{ADJ} nor *DRD4* genotype was associated with hippocampus volume in the initial models, and there were no CSI_{ADJ}-by-*DRD4* interaction effects (Table 2). When treatment and genotype were allowed to interact with age in the age-interaction models, however, a significant age-by-CSI_{ADJ}-by-*DRD4* interaction effect was found in the left hippocampus (p=0.008; β[CI]=0.323[0.086-0.561]; Figure 2). Irrespective of genotype, there was little association between CSI_{ADJ} and left hippocampal volume at older age, whereas at younger age a negative association was found in 7R-non-carriers and a positive association was found in 7R-carriers. The association was strongest in the 7R-carriers and at younger age. A similar but non-significant trend was found in the right hippocampus ($p_{AGE\text{-}BY\text{-}CSIADJ\text{-}BY\text{-}DRD4}$=0.066).

Age-by-ADHD-type-by-*DRD4* (i.e., replacing CSI_{ADJ} by ADHD-type) was not associated with left hippocampal volume. Finally, participants with and without ADHD did not differ in hippocampal volume (p_{LEFT}=0.838; p_{RIGHT}=0.277).

Alternative treatment parameters

In an attempt to disentangle treatment parameters contributing to CSI_{ADJ}, significant models were re-estimated replacing CSI_{ADJ} with non-adjusted cumulative dose, treatment duration, start age, current treatment (y/n), and time since last treatment. Age^2-by-treatment-by-*DRD4* interaction effects on the frontal cortex, significant when the treatment parameter was CSI_{ADJ}, were not significant when the treatment parameter was start age (p_{LEFT}=0.676, p_{RIGHT}=0.924), time since last treatment (p_{LEFT}=0.157, p_{RIGHT}=0.064), or current treatment (y/n) (p_{LEFT}=0.659, p_{RIGHT}=0.259). By contrast, when CSI_{ADJ} was replaced by non-adjusted cumulative intake or treatment duration, the effect changed very little (non-adjusted CSI: p_{LEFT}=0.003, p_{RIGHT}=0.001; duration: p_{LEFT}=0.009, p_{RIGHT}=0.002). When CSI_{ADJ} and treatment duration were modeled competitively, neither of the interaction terms reached significance, suggesting that the effects of CSI_{ADJ} and treatment duration at least partially overlap.

In the left hippocampus, replacing CSI_{ADJ} by non-adjusted cumulative dose, treatment duration, or start age yielded age-by-treatment-by-*DRD4* interaction effects similar to those of CSI_{ADJ}, but none reached significance according to the multiple comparisons threshold (p=0.013, p=0.014, and p=0.024, respectively). Current use and time since last treatment did not show such effects.

Discussion

We investigated associations between stimulant treatment and striatal, frontal, and hippocampal volumes, and potential moderating effects of genotype and age, in children, adolescents, and young adults with ADHD. There were three main findings. First, stimulant treatment was not associated with striatal volume. Second, associations between stimulant treatment and bilateral frontal and left hippocampal volume depended on *DRD4* genotype and

age. Associations were particularly pronounced in carriers of the *DRD4* 7R-allele at younger age, and were not accounted for by clinical and/or demographic confounders. Finally, irrespective of stimulant treatment, left striatal volume was reduced in carriers of one *DAT1* 10-6 risk allele compared to 10-6 homozygotes.

We had hypothesized that stimulant treatment would be associated with more normative regional brain volumes, especially at younger age and in carriers of *DAT1* 10-6 and/or *DRD4* 7R risk alleles. Striatal volumes were not altered in participants with ADHD compared to controls, which, as previously reported in the our sample,[3] may be due to volume reduction becoming less apparent at late-adolescent/early-adult age.[1,4] Furthermore, we found no indication of stimulant treatment positively affecting striatal volume, as had been suggested by two meta-analyses both including only slightly more participants compared to the current sample.[1,4] In contrast, our findings in the frontal cortex are consistent with age- and genotype-specific volumetric changes toward more normative levels after stimulant treatment. Frontal cortex volume was reduced in participants with ADHD compared to controls. In carriers of the *DRD4* 7R-allele, more intense stimulant treatment (either higher dose or longer duration) was associated with increased frontal cortex volumes at younger age. Such associations were not observed at older age, or in individuals not carrying the 7R-allele.

Although our observational study design is inconclusive as to whether associations between stimulant treatment and frontal cortex volume constitute treatment effects, it is worthwhile to explore possible underlying mechanisms. The frontal cortex of *DRD4* 7R-carriers may at younger age, e.g., in late childhood when D4 receptor density in the frontal cortex peaks,[48] exhibit postsynaptic characteristics allowing for long-term neural plasticity in the event of exposure to stimulants. Long-term plasticity occurs only when tonic dopamine levels, maintained by continuous background firing of dopaminergic neurons, are within an optimal range, i.e.,

neither too high nor too low.[49] Fine-tuning of tonic dopamine levels is managed through inhibitory feedback mechanisms involving D4 receptors[50] and has been associated with *DRD4* 7R-carriership.[51] Thus, early age, presence of the 7R-allele, and stimulant treatment may together adjust tonic dopamine levels to enable structural remodeling in the frontal cortex. Alternatively, the older participants may represent a specific patient population (i.e., persistent ADHD) that could be less likely to show genotype-by-treatment interaction effects, compared to the potentially more mixed younger group (i.e., this group likely includes participants who will remit during adolescence). This interpretation is plausible given the cross-sectional nature of our study. As another possibility, brain changes in 7R-carriers may result from an enhanced acute frontal cortex response to stimulant treatment at younger age, increasing the likelihood of long-term changes in this group. Finally, the lack of association between treatment and brain changes at later age may result from treatment discontinuation, i.e., lasting treatment effects may require ongoing treatment. Post-hoc analysis did not indicate significant contributions of current treatment (y/n) or time since last treatment, but individual and combined effects of various treatment parameters can only be disentangled in rigorously designed intervention studies. Note that speculations about potential micro-level mechanisms can only be tested using alternative approaches (e.g., animal or radio-ligand studies). Moreover, our findings await replication in an independent sample.

Similar to the frontal cortex findings, the association between stimulant treatment and left hippocampal volume was strongest in *DRD4* 7R-carriers and at younger age. Notably, there were no case-control differences in hippocampal volume. In 7R-carriers, especially at younger age, hippocampal volume appeared to deviate from the controls with more intense stimulant treatment. Similar but non-significant effects were found for treatment duration, non-adjusted cumulative dose, and start age, suggesting that these parameters each contribute to the effect of

CSI_{ADJ}. Treatment-related hippocampal volume reduction has previously been reported in individuals with ADHD.[52,53] Further investigation of long-term stimulant treatment effects on hippocampal development is warranted.

A final noteworthy finding was the larger striatal volume in *DAT1* 10-6 risk allele homozygotes compared to carriers of only one risk allele. At trend level, the non-carriers also differed from the heterozygotes, but not from the homozygotes, which could indicate a non-linear association (0 copies > 1 copy < 2 copies). Other studies have reported smaller striatal volumes in 3'UTR VNTR 10-repeat homozygotes compared to heterozygotes and/or non-carriers.[12,54] This seems at variance with the current findings, but note that these studies classified participants based on the 3'-UTR VNTR alone rather than on *DAT1* haplotype. Moreover, non-carriers were not examined in these previous studies. In follow-up analyses we found no association between striatal volume and the 3'-UTR 10/10 polymorphism alone (data not shown). Participants' age may also contribute to divergent findings; comparing striatal volumes of children/adolescents (including the current sample) and adults with different *DAT1* haplotypes, our group found that the 9-6 variant was associated with larger striatal volume in adults but not adolescents with ADHD.[55] It is noteworthy that in the current study we did not find this gene-by-age interaction effects on striatal volume, nor was the 9-6 variant associated with striatal volume (data not shown). Our finding of a putative non-linear pattern should not be over-interpreted; it did not reach our adjusted significance level, was not hypothesized *a priori*, and could not be related to existing literature. Replication of the *DAT1* haplotype findings in an independent sample is needed.

Essential features of the current study could not have been achieved in a randomized controlled design. Our sample covered a wide treatment- and age-range, allowing for the study of within-group heterogeneity. The focus on late-adolescence further allowed the investigation of

potential treatment effects occurring only after multiple years of treatment, and/or occurring long after treatment had been discontinued. Notwithstanding, the current observational design brings caveats regarding causal interference. Unmeasured pre-existing factors associated with treatment (e.g., pre-treatment symptom severity) or simultaneously occurring events (e.g., concurrent behavioral treatment) may have contributed to the observed associations. Second, age effects should be interpreted with appropriate caution given our cross-sectional design. Participants included at older age may represent a different population (e.g., persistent ADHD) compared to those included at younger age. A longitudinal study design is required to allow conclusions about developmental effects. Finally, we wish to emphasize that clinical application of our findings of small effect size is still several steps away, e.g., behavioral correlates of subtle brain changes require further investigation.

In sum, we investigated associations between stimulant treatment and regional brain volumes, and potential moderating effects of age and genotype, in a cross-sectional ADHD cohort. We found that frontal cortex volume was associated with stimulant treatment in carriers of the *DRD4* 7R-allele at younger age, possibly suggesting normalizing effects in these participants. Striatal volume was associated with *DAT1* haplotype, but not with treatment. We propose that neural sensitivity to long-term treatment effects may arise from genotype- and age-specific characteristics of postsynaptic dopamine receptors, allowing for long-term plasticity when exposed to stimulant treatment. The clinical relevance of subtle brain changes of small effect size is expected to be modest and requires further investigation; nevertheless, our findings may ultimately contribute to optimal care for individuals with ADHD.

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Table 1.

Demographic and clinical characteristics of the ADHD sample, and their associations with stimulant treatment.

			Association with CSI_{ADJ}[a]	
	n	%	F	r
Gender=male	219	69.3	29.8 *	
Site=Nijmegen	178	56.3	7.0	
Age (M,SD)	17.2	3.4		-0.01
IQ (M,SD)	96.1	16.1		-0.01
Socio-economic status (M,SD)	11.5	2.3		0.04
CPRS inattention (M,SD)	64.4	14.3		0.05
CPRS hyperactivity/impulsivity (M,SD)	68.2	17.4		0.05
DAT1 10-6 risk allele			3.6	
0 copies (non-carrier)	20	6.4		
1 copy (heterozygote)	134	42.7		
2 copies (homozygote)	160	51.0		
DRD4 7R risk allele			1.2	
0 copies (non-carrier)	208	65.8		
1 or 2 copies (carrier)	108	34.2		
ADHD-type			10.9 *	
Inattentive	141	44.6		
Hyperactive/impulsive	37	11.7		
Combined	138	43.7		

Comorbidity (any)	111	35.1	1.4	
History of stimulant treatment (y/n)	278	88.0	N/A	
Treatment duration in years (M,SD)	4.1	3.3		0.713 *
Start age (M,SD)	8.5	2.8		-0.496[b] *
Years since last treatment (M,SD)	1.5	2.3		-0.417[b] *
Currently on active treatment	148	46.8	59.8[b] *	
History of atomoxetine treatment (y/n)	39	12.3	0.5	
History of non-stimulant medication (y/n)	65	20.6	2.2	

* $p<0.008$; [a] age-adjusted cumulative stimulant intake; [b] within stimulant-treated (i.e., non-naïve) participants.

Table 2.

Regression weights of treatment, genotype, and treatment-by-genotype, and their interactions with age and age^2. Parameters of covariates and lower-level terms are available online.

	Striatum		Frontal cortex		Hippocampus	
	L	R	L	R	L	R
Initial model						
CSI_{ADJ}[a]	0.073	0.051	0.040	0.031	0.067	0.002
DAT1$_{0\text{-COPIES}}$	0.162	0.060				
DAT1$_{1\text{-COPY}}$	-0.252*	-0.186				
DRD4$_{\text{NON-CARRIER}}$			0.007	0.236	-0.001	0.023
CSI_{ADJ} x *DAT1*$_{0\text{-COPIES}}$	-0.170	-0.086				
CSI_{ADJ} x *DAT1*$_{1\text{-COPY}}$	-0.111	-0.081				
CSI_{ADJ} x *DRD4*$_{\text{NON-CARRIER}}$			-0.458	-0.240	-0.085	-0.008
Age-interaction model						
Age x CSI_{ADJ} x *DAT1*$_{0\text{-COPIES}}$	*ns*	*ns*				
Age x CSI_{ADJ} x *DAT1*$_{1\text{-COPY}}$	*ns*	*ns*				
Age x CSI_{ADJ} x *DRD4*$_{\text{NON-CARRIER}}$			0.756	0.458	0.158*	*ns*
Age^2 x CSI_{ADJ} x *DAT1*$_{0\text{-COPIES}}$	*ns*	*ns*				
Age^2 x CSI_{ADJ} x *DAT1*$_{1\text{-COPY}}$	*ns*	*ns*				
Age^2 x CSI_{ADJ} x *DRD4*$_{\text{NON-CARRIER}}$			-1.717*	-2.030*	*ns*	*ns*

[a] Age-adjusted cumulative stimulant intake.

Figure 1.

Participants' stimulant treatment trajectories. Daily stimulant dose (y-axis) is plotted as a function of age in years (x-axis), between age=0 and age at study participation. Participants are stratified in age quartiles; bold markers represent the average daily dose across participants within each quartile. Age at study participation was significantly associated with treatment duration (r=0.202), start age (r=0.298), and time since last treatment (r=0.377), and participants on active treatment are younger (M=15.9) compared to participants who had discontinued treatment (M=18.4).

Figure 2.

Three-way interaction effects between CSI_{ADJ}, *DRD4* genotype, and age or age^2, on volumes of the left and right frontal cortex and left hippocampus. For display, volumes based on the regression function are estimated at younger age (age=1SD below the mean, age^2=-1SD*-1SD) and at older age (age=1SD above the mean, age^2=+1SD*+1SD), and separately for carriers and non-carriers of the *DRD4* 7R-risk allele. In the absence of stimulant treatment in control participants, estimated average volume for controls is presented as a dashed horizontal line (+/-1SD shaded). CSI_{ADJ}=age-adjusted cumulative stimulant intake; mg/y = milligrams per year;